Protection of Chickens against *Escherichia coli* Infections by DNA Containing CpG Motifs†

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Synthetic oligodeoxynucleotides (ODN) containing CpG motifs (CpG-ODN) have been shown to be effective immunoprotective agents in murine models for a variety of viral, intracellular bacterial, and protozoan infections. Until now, the use of CpG-ODN to protect against extracellular bacterial infections has not been reported. The objective of this study was to investigate the effect of CpG-ODN against cellulitis and colibacillosis in broiler chickens, using a well-established model. At 22 days of age, birds received CpG-ODN by either the subcutaneous or intramuscular route. Three days later, a virulent isolate of *Escherichia coli* was applied to a scratch site on the caudal abdominal skin. Birds were examined for 10 days after the *E. coli* challenge, and pathological and bacteriological assessments were conducted on all birds. The control group of birds receiving no CpG-ODN$^{(2007)}$ had a survival rate of 15%. In contrast, groups that received CpG-ODN$^{(2007)}$, by either subcutaneous or intramuscular injection, had significantly higher survival rates ($P < 0.0001$). Furthermore, the size of the cellulitis lesion was significantly smaller in groups that received CpG-ODN$^{(2007)}$ by the subcutaneous route ($P < 0.01$). A dose of as little as 3.16 µg of CpG-ODN$^{(2007)}$, delivered 3 days prior to challenge by either the subcutaneous or intramuscular route, significantly protected birds against *E. coli* infection ($P < 0.01$). This study demonstrates that CpG-ODN$^{(2007)}$ has both local and systemic protective effects in broiler chickens. This is the first time that CpG-ODN$^{(2007)}$ has been demonstrated to have an immunoprotective effect against an extracellular bacterial infection in any food animal species.

The identification of events and signals that can boost immune responses to pathogens has been an active area of investigation for many decades. These studies have led to the discovery of various immunomodulators, as well as the cytokine pathways that allow cellular communication to direct the immune response. Initial studies demonstrated that specific DNA sequences from *Mycobacterium bovis* were effective in stimulating the production of cytokines and activating natural killer cells (28). These complementary studies clearly indicated that bacterial DNA had immunomodulatory effects. Furthermore, these immunostimulatory properties of bacterial DNA were shown to be due mainly to a specific sequence of cytosine-phosphodiester-guanine (CpG) motifs. They are present in the unmethylated state and occur at a higher frequency in bacterial DNA than in mammalian DNA (28). Other studies demonstrated that synthetic CpG-containing oligodeoxynucleotides (CpG-ODN) induce B-cell proliferation and immunoglobulin secretion, monocyte secretion of the Th1-like cytokines interleukin-12 and alpha, beta, and gamma interferons, and natural killer (NK) cell lytic activity (2, 4, 7, 12, 14, 19, 25, 27, 28, 30).

Furthermore, a number of studies indicate that CpG-ODN can shift the immune response to a Th1-dominated cytokine pathway. This explains the predominance of Th1-like responses when CpG-ODN are used as an adjuvant with subunit or conventional vaccines (6, 15, 31).

It is now well established that two general immune mechanisms function against pathogens. Innate immunity (antigen-nonspecific) recognizes molecular patterns unique to and characteristic of infectious organisms. Adaptive or acquired (antigen-specific) immunity uses T and B lymphocytes that express distinct receptors to recognize pathogen-derived peptides or epitopes. Innate immunity has been thought to provide only a rapid, short-term, incomplete antimicrobial host defense until the slower, antigen-specific immune response develops. Recently, it has been suggested that the innate immune response may play a pivotal role in immune regulation and the development of host immunity by identifying which antigens require a specific response and determining the nature of that response (1, 9, 22). These studies have led to the evolution of the "danger" hypothesis, which proposes that cells of the immune system can respond rapidly to bacterial invaders, allowing the host to clear the infection even before specific immune responses are developed (6, 13, 16–18).

Escherichia coli causes a variety of disease syndromes in poultry, including yolk sac infection, omphalitis, respiratory tract infection, swollen head syndrome, septicemia, and cellulitis (21, 26). Cellulitis, first reported in 1984 by Randall et al. (26) in England, refers to inflammation of the subcutaneous

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tissue and is typically seen in the lower abdomens and thighs of broiler chickens. No clinical signs have been associated with cellulitis in living birds, but the presence of the lesion results in the condemnation of part of or the entire carcass at processing. Cellulitis has been reproduced in broilers by damaging the surface of the skin and applying virulent *E. coli* strains of serogroup O78 or O2 (10, 23, 24). A percentage of experimentally infected birds go on to develop colibacillosis, which is characterized in its acute form by septicemia resulting in death and in its subacute form by pericarditis, airsacculitis, and perihepatitis (5). The pathogenesis of colibacillosis is poorly understood, but the development of bacteremia appears to be essential (5, 20).

Although CpG-ODN have been used in laboratory animals and primates as inducers of innate immunity and as a vaccine adjuvant, their activity in chickens has not been reported to date. The objective of this study was to determine the effect of CpG-ODN as immunostimulants in broiler chickens by using an *E. coli* infection model.

MATERIALS AND METHODS

Bacteria. A field isolate of *E. coli* serogroup O2 from a turkey with colisepticemia was used as the source of the challenge strain. This *E. coli* strain was nonhemolytic, was serum resistant, produced aerobactin, and had a K1 capsule and type I pili. Aliquots of this field isolate were stored at −70°C in brain heart infusion broth (BHI) (Difco, Detroit, Mich.) supplemented with 25% (wt/vol) glycerol (VWR Scientific Inc., Montreal, Quebec, Canada). Cells derived from a frozen culture were passaged once on sheep blood agar and then grown aerobically at 37°C in BHI for 12 to 14 h in 50-ml glass flasks with shaking at 200 rpm. A 1:10,000 dilution of the culture then was made in a 1-liter flask containing 100 ml of BHI. This was incubated for 8 to 9 h under the conditions described above until an absorbance of 0.8 at 600 nm was reached. This is known to produce a culture containing approximately 10^9 CFU per ml in the logarithmic phase of growth. The actual number of bacteria was determined by plating duplicate 10-fold serial dilutions of the culture on sheep blood agar and incubating at 37°C for 24 h. The challenge inoculum was washed once in normal saline and resuspended to an absorbance of 0.8 at 600 nm.

Synthetic CpG-ODN. The sequence of CpG-ODN[(2007)] was TCGTCGTTGT CGTTTTGTCGTT, and the sequence of non-CpG-ODN[(2041)] was CTGGTCT TTCTGGTTTTTTTCTGG (underlining indicates CpG dinucleotides). Both ODN were produced with a phosphorothioate backbone. Synthetic CpG-ODN were diluted in sterile, pyrogen-free saline and administered in a 1-ml volume by either the subcutaneous or intramuscular route.

Animal model. All procedures involving animals were done with the approval of the University of Saskatchewan Committee on Animal Care. Day-old broiler chicks were obtained from a local hatchery in Saskatchewan, Canada, and identified individually by wing bands. Groups of chicks were randomly allocated into individual animal isolation rooms at the Veterinary Infectious Disease Organization, University of Saskatchewan. Water and commercial broiler ration were provided ad libitum, and a photoperiod of 8 h per 24 h was established. Room temperature was maintained at 20 to 22°C. Each room was ventilated with filtered, nonrecirculated air at a rate of 10 to 12 changes/h. Air pressure differentials and strict sanitation were maintained in this isolation facility. The model used for the production of cellulitis and colibacillosis in broiler chickens was a modification of one previously described (11). At day 25 of age, two parallel 3-cm-long, full-thickness scratches were made through the skin on the left caudal abdominal region with a diabetic lancet (Lifescan Lancets; Lifescan Canada Ltd., Burnby, British Columbia, Canada). The challenge inoculum was applied with two cotton applicators (Hardwood Products Company LLC, Guilford, Maine) to the scratches. Application of *E. coli* was repeated 15 min later. In this model, cellulitis develops in 100% of birds, and pericarditis, airsacculitis, and perihepatitis develop in 50 to 90% of birds. Occasionally, a few birds also develop hepatitis. Mortality ranges between 50 and 90% of the birds.

Birds were observed twice daily for 9 days postchallenge for clinical signs, and each individual was assigned daily clinical scores as follows: 0, normal; 0.5, slightly abnormal appearance, slow to move; 1, depression, reluctant to move; 2, inability to stand or reach food or water; and 3, dead. All of the birds were scored

daily for 9 days postchallenge. Birds that received a clinical score of 2 were euthanized by cervical dislocation.

Chickens that were found dead or were euthanized were necropsied immediately. Gross pathological examinations, including the measurement of the size of the cellulitis lesion, and bacteriological cultures were done. Bacterial swabs were taken from the cellulitis lesion, pericardium, and air sacs and cultured on MacConkey agar (Difco) and tryptic soy agar with sheep blood (PML Microbiologicals, Richmond, British Columbia, Canada). For isolation of *E. coli*, a semiquantitative estimate of *E. coli* was conducted on MacConkey agar (3). Any gross lesions were recorded. On day 10, the remaining birds were euthanized by cervical dislocation, and necropsy and bacteriological procedures were done as described above.

Experimental design. (i) Effect of CpG-ODN[(2007)] treatment on *E. coli* infection in chickens. This large-scale experiment was designed after a preliminary experiment, using 80 birds, indicated a protective effect of CpG-ODN against cellulitis and colibacillosis. Two hundred birds were randomly allocated into five groups of 40 birds each, identified by wing band, injected with CpG-ODN[(2007)] on day 22, and randomly distributed to 10 isolation rooms. Two groups received either 10 or 50 μg of CpG-ODN[(2007)] in the subcutaneous tissues of the left caudal abdomen. Two other groups received either 10 or 50 μg of CpG-ODN[(2007)] intramuscularly in the left drumstick. Half of the control group received saline intramuscularly in the left drumstick, and the remaining birds in the control group received saline subcutaneously in the left caudal abdomen. All of the groups were challenged on day 25 with *E. coli* as described above. Clinical assessments, necropsy examinations, and bacteriology were done as previously described.

(ii) Dose titration of CpG-ODN[(2007)] by the subcutaneous route. Birds were randomly allocated into seven groups of 20 birds each, identified by wing band, and injected with CpG-ODN[(2007)] on day 22. Four groups of birds received 100, 31.6, 10, or 3.16 μg of CpG-ODN[(2007)] subcutaneously in the left caudal abdomen. To determine if the effect of CpG-ODN treatment was localized, one group of birds received 31.6 μg of CpG-ODN[(2007)] in the subcutaneous tissues of the neck. The control group received saline in the subcutaneous tissue of the left caudal abdomen. All of the groups were challenged on day 25 with *E. coli* as described above. Clinical, bacteriological, and gross pathological evaluations were conducted as in the previous experiment.

(iii) Dose titration of CpG-ODN delivered by the intramuscular route. Birds were randomly allocated into eight groups of 24 birds, wing banded, randomly distributed into isolation rooms, and injected intramuscularly with CpG-ODN[(2007)] on day 22. Four groups of birds received 100, 31.6, 10, or 3.16 μg of CpG-ODN[(2007)] in the left drumstick. Three negative control groups received normal saline or 10 or 3.16 μg of non-CpG-ODN[(2041)] in the left drumstick. All of the groups were challenged on day 25 with *E. coli*. Clinical, bacteriological, and gross pathological evaluations were conducted as described above.

(iv) Duration of protection against *E. coli* infection by administration of CpG-ODN[(2007)]. Birds were randomly allocated into five groups of 24 birds, wing banded, randomly distributed into isolation rooms, and injected with 10 μg of CpG-ODN[(2007)] subcutaneously in the left caudal abdomen on either day 10, 13, 16, 19, or 22 of age. All of the groups were challenged on day 25 with *E. coli* as described above. Clinical, bacteriological, and gross pathological evaluations were conducted as described above.

(v) Evaluation of tissue reactions at the site of CpG-ODN administration. Fourteen-day-old birds were randomly allocated into seven groups of 15 birds each. Two groups of birds received CpG-ODN[(2007)] (100 or 3.16 μg) subcutaneously in the left caudal abdomen and saline in the right caudal abdomen. Two groups of birds received CpG-ODN[(2007)] (10 or 3.16 μg) in the left drumstick and saline in the right drumstick. Two groups of birds received 3.16 μg of non-CpG-ODN[(2041)] subcutaneously in the left caudal abdomen or intramuscularly in the left drumstick and received saline in either the right caudal abdomen or drumstick accordingly. A control group of birds were injected subcutaneously with saline in the right caudal abdomen and right drumstick. Three birds were euthanized on each of days 1, 3, 7, 10, and 14 postinoculation, and tissue sections were taken for histopathology. Tissue sections from the caudal abdominal subcutaneous tissue or drumstick were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-μm thickness and stained with hematoxylin and eosin. A histological score from 0 to 4 (0, no visible lesions; 1, focal, mild mononuclear, and heterophil infiltrations or cellulitis or myositis; 2, locally extensive, moderate mononuclear and heterophil infiltrations or cellulitis or myositis; 3, locally extensive, moderate to severe mononuclear and heterophil infiltrations or cellulitis or myositis; 4, diffuse, severe mononuclear and heterophil infiltrations or cellulitis or myositis) was assigned based on the tissue reactions to the various doses of CpG-ODN.
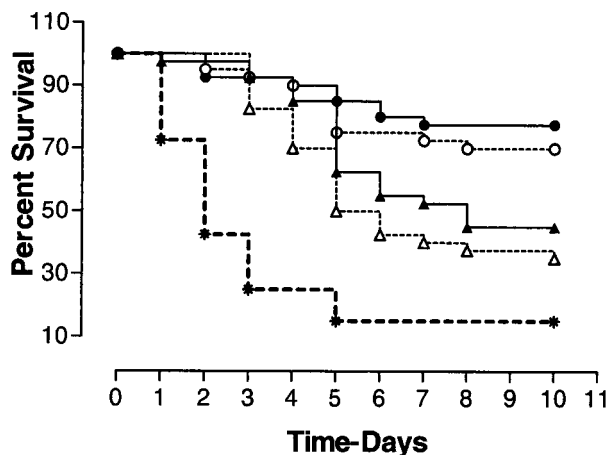
FIG. 1. Survival of chickens following various treatments of CpG-ODN[(2007)]. Groups of birds were treated subcutaneously (circles) or intramuscularly (triangles) with 50 µg (closed symbols) or 10 µg (open symbols) of CpG-ODN[(2007)], or with saline as a control (asterisks), 3 days prior to exposure to *E. coli*. Each group consisted of 40 birds. CpG-ODN[(2007)] treatment significantly protected against *E. coli* infection compared to the level of infection in the control group ($P < 0.0001$).

(vi) *E. coli* septicemia in birds treated with CpG-ODN[(2007)]. Birds were randomly allocated into two groups of 20 birds, wing banded, randomly distributed into isolation rooms, and injected with 50 µg of CpG-ODN[(2007)] subcutaneously in the left caudal abdomen on day 22 of age. All of the birds were challenged on day 25 with *E. coli* as described above. One milliliter of blood was collected from all of the birds into 3-ml syringes containing heparin (Organon Teknika, Toronto, Ontario, Canada) on days 1, 2, 4, 7, and 8 postchallenge with *E. coli*. The actual number of bacteria in the blood sample was determined by plating 10-fold serial dilutions in duplicate on sheep blood agar and MacConkey agar (Difco) and incubating at 37°C for 24 h. Clinical evaluation and gross pathological evaluations were conducted as described above.

Statistical analysis. Comparisons of survival patterns and median survivals were done by the log rank test. The significance of treatment effects on the size of cellulitis lesions was determined by one-way analysis of variance (nonparametric, Kruskal-Wallis) and the *t* test (Mann-Whitney). The significance of effect of treatment group on *E. coli* isolation score was determined by contingency table analysis and the chi-square test or Fisher's exact test as appropriate. All data were analyzed by using Prism 3.0 (GraphPad Software Inc., San Diego, Calif.), with a *P* of 0.05 indicating significance.

RESULTS

Effect of CpG-ODN[(2007)] treatment on *E. coli* infection in chickens. All of the birds in the experiment developed cellulitis following exposure to *E. coli*. Survival in all CpG-ODN[(2007)]-treated groups was significantly better than that in the control group ($P < 0.0001$) (Fig. 1). Treatment of birds with CpG-ODN[(2007)] by subcutaneous injection significantly increased survival compared to treatment with CpG-ODN[(2007)] by intramuscular injection ($P < 0.01$). The majority of the birds in the control group showed clinical signs of disease compared to groups treated with CpG-ODN[(2007)]. Birds treated with CpG-ODN[(2007)] subcutaneously had the fewest clinical signs. Furthermore, the size of the cellulitis lesion was significantly smaller in birds treated subcutaneously (10 µg, 4.4 ± 3.0 cm^2; 50 µg, 5.7 ± 3.7 cm^2) than in the control group (8.5 ± 3.7 cm^2) ($P < 0.0001$). In contrast, the size of the cellulitis lesion was not different between the control group and the group treated intramuscularly (10 µg, 8.6 ± 2.9 cm^2; 50 µg, 8.9 ± 3.3 cm^2)

TABLE 1. Bacterial isolation from different lesions

Lesion site[a]	*E. coli* growth score[c]	No. of birds in treatment group[b]:				
		Control	I/M, 10 µg	I/M, 50 µg	S/C, 10 µg	S/C, 50 µg
Cellulitis	0	4	6	9	18	19
	1+	4	14	9	11	13
	2+	7	8	11	4	1
	3+	15	11	6	6	6
	4+	10	1	5	1	1
Pericardium	0	10	13	16	27	30
	1+	4	4	5	3	1
	2+	6	7	4	5	2
	3+	13	18	11	4	3
	4+	7	3	4	1	4
Air sacs	0	8	14	16	28	31
	1+	7	8	4	1	0
	2+	8	4	5	5	3
	3+	14	12	9	4	5
	4+	3	2	6	2	1

[a] Bacterial isolations from the cellulitis lesions, air sacs, and pericardiums, of birds in the large-scale experiment (see Materials and Methods).

[b] Birds were treated with 10 or 50 µg of CpG-ODN[(2007)] by either the subcutaneous (S/C) or intramuscular (I/M) route.

[c] The *E. coli* concentration in lesions was estimated on MacConkey agar by streaking sequentially in four distinct areas on agar plates. The enumeration was expressed on a scale of 0 to 4+ (0, no growth; 1+, growth of bacteria on area 1; 2+, growth of the bacteria on areas 1 and 2; 3+, growth of bacteria on areas 1, 2, and 3; 4+, growth of bacteria on areas 1, 2, 3, and 4). The effect of treatment on *E. coli* growth was very significant ($P < 0.01$). Heavy bacterial growth was observed more frequently in lesions from birds in the control group or groups treated with CpG-ODN[(2007)] by the intramuscular route than in the groups treated with CpG-ODN[(2007)] by the subcutaneous route.

with CpG-ODN[(2007)] ($P > 0.05$). The majority of birds in the control group developed airsacculitis, pericarditis, and perihepatitis, either alone or in combination (polyserositis), in addition to cellulitis. Fewer birds developed similar lesions in the groups treated with CpG-ODN[(2007)] subcutaneously. Occasionally, some birds developed multifocal hepatitis. Birds which died peracutely within 24 h of challenge did not have gross lesions, but many bacteria were cultured from the air sacs, pericardium, and abdominal inoculation sites. In groups treated with CpG-ODN[(2007)] subcutaneously, bacteria were isolated less frequently from the cellulitis lesion, pericardium, and air sacs than in the control or intramuscularly CpG-ODN[(2007)]-treated groups (Table 1).

Dose titration of CpG-ODN[(2007)] by the subcutaneous route. Since the subcutaneous administration of CpG-ODN was most effective, a second trial was conducted to evaluate the effect of dosage by this route. All of the groups given CpG-ODN[(2007)] subcutaneously in the left caudal abdomen had significantly better survival than the control group ($P < 0.01$) (Fig. 2). In contrast, birds that had received CpG-ODN[(2007)] in the neck did not have increased survival ($P > 0.05$). The pattern of clinical signs and bacterial culture results were similar to those observed in the previous experiment. The cellulitis lesions were significantly smaller in birds treated subcutaneously in the caudal abdomen with CpG-ODN[(2007)] (100, 31.6, and 3.16 µg) than in the control group ($P < 0.05$). The size of the cellulitis lesion in birds treated with CpG-ODN[(2007)] (31.6 µg) in the
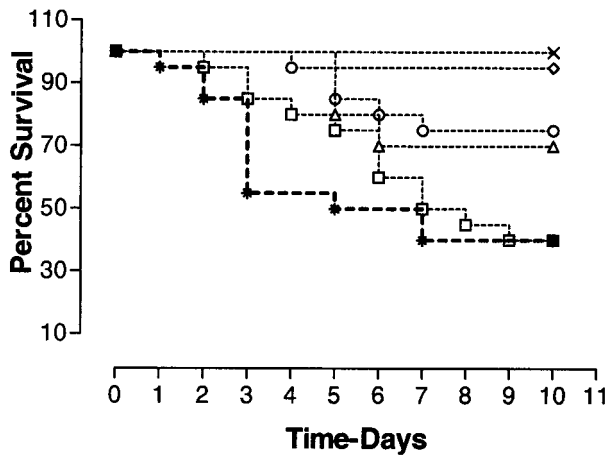
FIG. 2. Survival of chickens following various doses of CpG-ODN[(2007)] administered by the subcutaneous route. Groups of birds ($n = 20$) were administered doses of CpG-ODN[(2007)] of 3.16 μg (◇), 10 μg (○), 31.6 μg (×), and 100 μg (△) per bird in the caudal abdomen or of 31.6 μg per bird in the neck (□), by subcutaneous injection prior to infection with *E. coli*. All of the groups treated with CpG-ODN[(2007)] in the caudal abdomen were significantly protected against *E. coli* infection compared to the control group (*) ($P < 0.01$). The group of birds given CpG-ODN[(2007)] subcutaneously in the neck to study the local effect of CpG-ODN[(2007)] was not protected against *E. coli* infection ($P > 0.05$).

neck was not significantly different ($P > 0.05$) from that in the control group.

Dose titration of CpG-ODN delivered by the intramuscular route. Since the intramuscular administration of CpG-ODN significantly protected birds against *E. coli* infections, a third trial was conducted to evaluate the effect of dosage by this route. Each group of birds that received CpG-ODN[(2007)], at doses ranging from 100 to 3.16 μg, had significantly increased
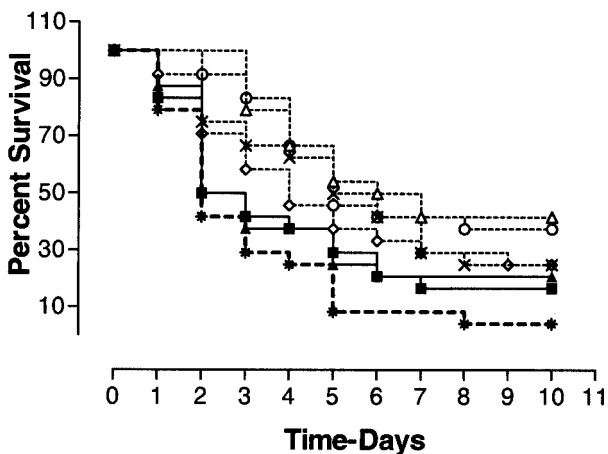

FIG. 3. Survival of chickens given various doses of CpG-ODN[(2007)] by the intramuscular route. Groups of birds ($n = 20$) were administered doses of CpG-ODN[(2007)] of 3.16 μg (◇), 10 μg (○), 31.6 μg (×), and 100 μg (△) per bird or non-CpG-ODN[(2041)] at 3.16 μg (▲) or 10 μg (■) per bird by intramuscular injection 3 days prior to infection with *E. coli*. All groups treated with CpG-ODN[(2007)] were significantly protected against *E. coli* infection compared to the control group (*) ($P < 0.01$). Groups treated with non-CpG-ODN[(2041)] were not protected against *E. coli* infection ($P > 0.05$).
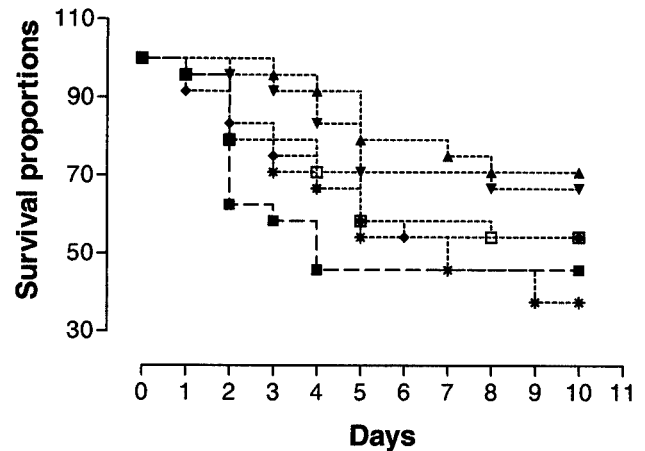

FIG. 4. Duration of protection induced by CpG-ODN. Chickens were treated with 10 μg of CpG-ODN[(2007)] by the subcutaneous route on day 10 (□), 13 (*), 16 (◆), 19 (▼), or 22 (▲) and challenged with *E. coli* on day 25 ($n = 24$). Birds were significantly protected ($P < 0.01$) by administration of CpG-ODN[(2007)] 3 days prior to challenge with *E. coli*, and the protection decreased with increasing interval between treatment and challenge. ■, control.

survival compared to the control group ($P < 0.01$) (Fig. 3). The groups that received non-CpG-ODN[(2041)] did not show increased survival after *E. coli* infection ($P > 0.05$). The pattern of clinical signs and bacterial isolations were similar to those in the previous experiment. The size of the cellulitis lesion was not significantly different in birds treated with CpG-ODN[(2007)] by intramuscular injection from that in the control group ($P > 0.05$).

Duration of protection against *E. coli* infection by administration of CpG-ODN[(2007)]. Birds were treated with CpG-ODN at different times prior to challenge to determine the duration of the protective effect. The group which received CpG-ODN[(2007)] 3 days prior to challenge had significantly better survival than the control group ($P < 0.05$) (Fig. 4). Although mortality was reduced, survival in the group that received CpG-ODN[(2007)] 6 days prior to challenge was not significantly improved ($P = 0.06$). The groups that received CpG-ODN[(2007)] from 9 to 15 days prior to challenge did not show increased survival after *E. coli* infection ($P > 0.05$). The pattern of clinical signs and bacterial isolations were similar to those in the previous experiment.

Evaluation of tissue reactions at the site of CpG-ODN administration. Since some of the effect of CpG-ODN administration appeared to be localized, a study was conducted to examine the tissue response at the site of injection (Fig. 5). In tissues that received CpG-ODN[(2007)] by subcutaneous injection, cellular infiltration was mild to severe for up to 10 days but had completely resolved by day 14 for the 3.16-μg treatment. In contrast, cellular infiltration was severe on day 1 and completely resolved by day 3 in birds that received 0.1 μg (Table 2).

Cellular infiltration was detected up to day 14 after intramuscular injection of 10 μg of CpG-ODN[(2007)] but was mild to moderate until day 7 and had completely disappeared by day 10 for the 3.16-μg treatment.

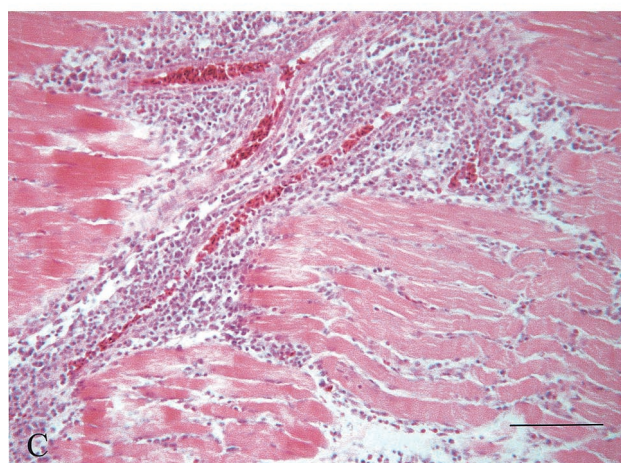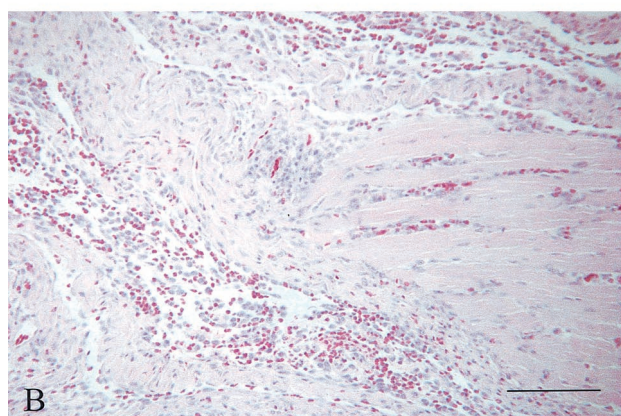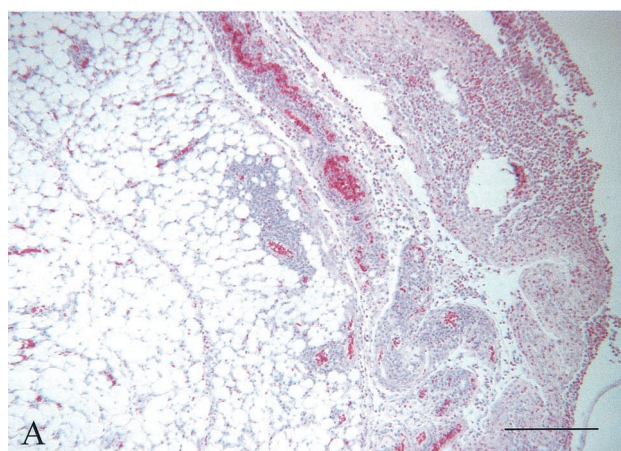To determine the contribution of the CpG motif to the

FIG. 5. Histopathological evaluation of subcutaneous tissues of caudal abdomens of birds treated with CpG-ODN[(2007)]. Tissues were taken 24 h after inoculation of CpG-ODN[(2007)], fixed in 10% neutral buffered formalin, sectioned at 5-μm thickness, and stained with hematoxylin and eosin. Both heterophils and mononuclear cells were infiltrated in the subcutaneous tissue and muscles without causing tissue necrosis. (A) Low magnification to demonstrate cellular infiltration in the subcutaneous tissue at 24 h after administration of CpG-ODN. Bar, 200 μm. (B) Higher magnification of panel A to demonstrate predominant heterophils (red). Bar, 100 μm. (C) Demonstration of predominant mononuclear cells (blue) in muscle tissue after 72 h of administration of CpG-ODN. Bar, 100 μm.

TABLE 2. Histological changes in birds following administration of CpG-ODN[a]

ODN	Dose (μg/bird)	Route(s)	Gross pathology score[b] on day:				
			1	3	7	10	14
CpG-ODN[(2007)]	3.16	S/C	3	3	1	1	0
	0.1	S/C	3	0	0	0	0
	10	I/M	3	3	3	3	1
	3.16	I/M	1	2	2	0	0
Non-CpG-ODN[(2041)]	3.16	S/C	2	1	0	0	0
	3.16	I/M	0	0	0	0	0
Control (saline)		I/M and S/C	0	0	0	0	0

[a] Histopathological score (cellular infiltration) following administration of CpG-ODN by either subcutaneous (S/C) or intramuscular (I/M) injection. Cellular infiltration was most pronounced and persisted longest in birds treated with 10 μg of CpG-ODN[(2007)] by the intramuscular route.
[b] 0, no visible lesions; 1, focal, mild mononuclear and heterophil infiltrations; 2, locally extensive, moderate mononuclear and heterophil infiltrations; 3, locally extensive, moderate to severe mononuclear and heterophil infiltrations.

inflammation, some birds received the non-CpG-ODN[(2041)]. Treatment with this ODN by subcutaneous injection resulted in mild to moderate cellular infiltration at 3 days that had completely resolved by day 7 (Table 2). There was no cellular infiltration in response to treatment with 3.16 μg of non-CpG-ODN[(2041)] by intramuscular injection. No cellular infiltration was observed in any of the saline-injected sites.

***E. coli* septicemia in birds treated with CpG-ODN[(2007)].** To evaluate the systemic protective effect of CpG-ODN, the level of bacteremia was monitored following challenge. *E. coli* was isolated in the control group at a higher frequency than in the group that received CpG-ODN[(2007)] ($P < 0.05$) (Table 3), and the relative risk parameter indicated that 59% of birds that received this treatment became bacteremic, compared to controls. Also, the birds that received CpG-ODN[(2007)] had a lower number of *E. coli* than the control group ($P < 0.01$). Treatment also delayed the development of bacteremia. In the control group, of the 16 birds in which bacteremia was detected, 14 of those had *E. coli* isolated on day 1. In contrast, it was not until day 4 postchallenge that bacteremia was first detected in five of the nine birds which developed bacteremia in the CpG-ODN[(2007)]-treated group. The group that received CpG-ODN[(2007)] had significantly increased survival compared to the control group ($P < 0.01$), and the pattern of clinical signs and bacterial isolations from the internal lesions were similar to those in the previous experiments.

DISCUSSION

It is generally accepted that disease prevention by prophylactic measures or by immune stimulation is preferred to the use of therapeutic agents in the food animal industry. This has become especially important due to a number of food safety and human health issues. For example, the use of antibiotics by the animal industry has been linked to the emergence of resistant strains of bacteria. Also, the possibility of drug residues in meat products is an important issue, especially for international trade. For these reasons, the animal production industry is seeking to reduce the use of antibiotics. Novel immunostimulants may serve as alternative disease-preventive agents.

TABLE 3. *E. coli* septicemia in birds challenged with *E. coli* following administration of 50 μg of CpG-ODN[(2007)] subcutaneously[a]

Group	Bird no.	CFU of *E. coli*/ml on day:				
		1	2	4	7	8
Control	1	0	0	0	0	0
	2	2×10^5	Dead			
	3	5×10^2	7×10^2	Dead		
	4	0	2×10^3	0	0	0
	5	Dead				
	6	0	0	0	0	0
	7	1×10^5	Dead			
	8	1×10^8	Dead			
	9	1×10^4	1×10^4	Dead		
	10	1×10^5	1×10^3	Dead		
	11	1×10^3	3×10^2	1×10^2	0	0
	12	4×10^5	Dead			
	13	1×10^4	Dead			
	14	6×10^4	Dead			
	15	1×10^5	1×10^5	Dead		
	16	1×10^4	2×10^3	Dead		
	17	7×10^2	2×10^2	Dead		
	18	0	1×10^5	0	0	0
	19	2×10^2	Dead			
	20	0	0	0	0	0
Treatment	21	0	0	2×10^4	0	0
	22	0	0	0	0	0
	23	0	0	1×10^3	Dead	
	24	0	0	7×10^4	1×10^6	Dead
	25	2×10^2	6×10^2	Dead		
	26	0	0	0	0	0
	27	0	0	0	0	0
	28	0	4×10^2	Dead		
	29	0	0	0	0	0
	30	0	0	0	0	0
	31	0	6×10^3	Dead		
	32	0	0	1×10^2	0	0
	33	0	0	0	0	0
	34	0	0	0	0	0
	35	0	0	0	0	0
	36	0	0	0	0	0
	37	0	0	1×10^2	0	0
	38	0	0	0	0	0
	39	2×10^3	1×10^4	Dead		
	40	0	0	0	0	0

[a] CFU of *E. coli* in peripheral blood of birds treated with CpG-ODN[(2007)] and a control group on days 1, 2, 4, 7, and 8 following *E. coli* challenge ($n = 20$). *E. coli* was isolated on both sheep blood and MacConkey agar. The majority of birds in the control group had *E. coli* isolations on days 1 and 2 following *E. coli* challenge. CpG-ODN[(2007)] treatment significantly protected against *E. coli* infection compared to the level of infection in the control group ($P < 0.01$).

Synthetic CpG-ODN have been shown to be effective stimulators of the immune system and potent adjuvants in a number of species, including mice, primates, and humans (8, 29). However, this study demonstrates, for the first time, that CpG-ODN are efficacious as immunostimulants in poultry, a food animal species. Furthermore, while CpG-ODN have been effective in controlling intracellular protozoa, bacteria, and viruses, this is the first report showing CpG-ODN to be efficacious against a bacterial poultry disease, in which the bacteria do not have an intracellular phase.

The mortality rate in our animal model was generally above 50% and was associated with septicemia (11) and the development of systemic and local lesions. Our experiments demonstrated enhanced survival of birds that received CpG-ODN, by either the intramuscular or subcutaneous route, in a dose-dependent manner. In all instances, subcutaneous administration was more effective than intramuscular administration of CpG-ODN. Similarly, clinical disease scores, number of gross lesions, and amount of bacteria isolated from lesions were highest in the control group, intermediate in the group that received CpG-ODN intramuscularly, and lowest in the group that received CpG-ODN by subcutaneous injection. The mortality, clinical scores, and development of gross lesions are more severe in this model than in natural outbreaks of *E. coli* infections, where mortality is lower and subclinical infections cause major economic losses due to poor feed conversion and growth rate. Since CpG-ODN were able to significantly reduce the mortality in this severe model, we believe that CpG-ODN would be able to control naturally occurring, less severe *E. coli* infections.

Based on the histological data, doses of as low as 0.1 μg of CpG-ODN recruited both mononuclear and polymorphonuclear cells to the site of administration. Since we administered CpG-ODN and infected birds with *E. coli* at the same location in the subcutaneous groups, it is likely that inflammatory cells, recruited prior to challenge, reduced the number of bacteria that were available to enter systemic circulation. This local effect may explain why the administration of CpG-ODN by intramuscular injection resulted in a lower survival rate than the administration of CpG-ODN by the subcutaneous route. This explanation is supported by the observation that the administration of CpG-ODN by subcutaneous injection at a distant location (the neck) did not increase the survival rate (Fig. 2). The smallest cellulitis lesions were observed in birds that received CpG-ODN by the subcutaneous route, compared to either the control, intramuscular, or intravenous route. This observation also indicates that some bacteria are killed at the site of the CpG-ODN administration before they can establish the lesion.

Although there was some cellular infiltration to the site of non-CpG-ODN administration, cell recruitment was augmented by the presence of the CpG motif, demonstrating that similar motifs are recognized in chickens as in other species. Although this cellular infiltration was very high, occurred within 24 h, and lasted for up to 14 days, we did not see any indication of necrosis or cell death at the site of infiltration. This observation has also been reported for mice, and it was suggested that CpG-ODN failed to activate mast cell degranulation (30) and hence caused no tissue necrosis. Immunostimulation by CpG-ODN without causing tissue necrosis at the injection site would be a tremendous advantage to the poultry industry, since tissue damage due to injections leads to condemnation or trimming of parts of the carcass at slaughter and causes significant reduction of meat quality.

As well as having a local effect on the development of the cellulitis lesion, CpG-ODN treatment also had systemic effects as demonstrated by protection by intramuscular injection. These systemic effects were also demonstrated in that CpG-ODN treatment was able to minimize the incidence and the level of, and delay the development of, septicemia.

Although microbial contamination of poultry meat is controlled by modern sanitary slaughter procedures, bacteria commonly are found in retail poultry products. Reduction of the amount of bacteria present in the carcass is one way to reduce the risk that the meat products will become contaminated. We

have shown that treatment with CpG-ODN reduced the amount of *E. coli* bacteria present in lesions of cellulitis, pericarditis, and airsacculitis. Reducing the amount of *E. coli* in the bird reduces the risk that poultry meat will become contaminated with potentially hazardous bacteria during processing and thus will improve food safety. This is especially important for those poultry processors that trim rather than condemn poultry with cellulitis.

CpG-ODN treatment was consistently effective when given 3 days prior to infection and appeared to have some effect for almost 6 days (Fig. 4). This duration of effect represents a significant proportion of the life span of a broiler chicken, which is from 35 to 42 days. Thus, work on the effect of repeated administration and development of feasible delivery methods such as aerosol or oral delivery are necessary to determine the utility of CpG-ODN use in the poultry industry. Moreover, *E. coli* infection-associated mortality in the first week of life is a significant problem. Currently, antibiotics are used as a preventative measure, but there is the potential to use CpG-ODN in ovo to reduce the incidence of *E. coli* infection in the first week of life.

In conclusion, CpG-ODN were effective immunoprotective agents in poultry, as in other species. Further research into the mechanism of action and testing in other poultry disease models is required to determine how the recognition of CpG motifs by the avian immune system compares to that by other species and how utility of these immunostimulatory agents in the poultry industry can be developed.

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